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Hepatitis C Genotypes in Patients With Dual Hepatitis B and C Virus Infection

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In patients with chronic hepatitis B and C virus (HBV, HCV) infection, an inverse relationship in the replicative activity of the two viruses has been reported. In the present study the genotype of HCV was evaluated in 34 consecutive cases found with hepatitis B surface antigen (HBsAg) and anti-HCV in the serum, in order to identify its possible influence in determining the pattern of HBV/HCV interaction. Nineteen patients were HCV-RNA positive and could be genotyped: 8 were infected by HCV-1 (3 by HCV-1a and 5 by HCV-1b), 10 by HCV-2, and only 1 by HCV-3. Among these, 3 were HBV-DNA positive, compared to 10 of 15 HCV-RNA-negative patients ($P = 0.003$), and all 3 were coinfected with HCV-2.

Mean alanine aminotransferase (ALT) levels were similar between patients infected with HCV-1 and HCV-2. Among 7 patients with cirrhosis 5 were infected by HCV-2, while 6 of 12 of those without cirrhosis had HCV-1 infection.

In conclusion, HBV replication was inhibited more efficiently by HCV-1 than by HCV-2. Cirrhosis was frequently found in patients with dual HBV and HCV-2 infection.

KEY WORDS: chronic hepatitis, multiple virus infection, viral interference

INTRODUCTION

Following the discovery of hepatitis C virus (HCV) as the major cause of chronic liver disease worldwide and due to the availability of specific diagnostic markers to identify infection, several studies have been undertaken to evaluate the interaction between the hepatitis B (HBV) and C (HCV) viruses in patients with chronic hepatitis and cirrhosis since concurrent infection by the two agents may influence the severity and progression of liver disease. It has been reported that between 8% [Fattovich et al., 1991; Benvegnù et al., 1994] and 18% [Sato et al., 1994] of chronic hepatitis B surface antigen (HBsAg) carriers are also infected by HCV, and an inverse relationship in the replicative activity of the two viruses in individual patients has been described [Liaw et al., 1991, 1994; Sheen et al., 1992; Pontisso et al., 1993; François et al., 1993].

In order to investigate whether the genotype of HCV has any influence in determining the pattern of HBV/HCV interaction, the HCV type and subtype were examined in 34 patients with dual HBV and HCV infection in relation to the expression and replication of the two viruses and the severity of the associated liver disease.

PATIENTS AND METHODS

Patients

The patients were 34 consecutive cases of chronic liver disease found with HBsAg and anti-HCV in serum as determined by enzyme immunoassay (EIA) and RIBA-2 testing. All patients were tested for serum HCV-RNA by nested PCR using a screening procedure starting directly from 3 μl of serum or, in those negative by this procedure, by nested PCR after nucleic acid extraction from 100 μl of serum (see below). In patients with detectable serum HCV-RNA sequences by either method, the genotype and subtype of HCV were determined. All the patients were also tested for serum HBV-DNA by spot hybridisation.

Sera from 49 consecutive anti-HCV-positive patients without HBV infection were used as controls. None of the patients was treated with interferon at the time of the study.

Viral Markers

Serum HBsAg, anti-HBc, HBeAg and anti-HBe, anti-HDV were examined by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Abbott Diagnostics, North Chicago, IL). Anti-HCV was studied by second-generation ELISA (Ortho Diagnostics, Raritan, NJ) and confirmed by RIBA-2 (Chiron Corporation, Emeryville, CA).

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Serum HBV-DNA

Serum HBV-DNA was evaluated by spot hybridisation using a 32-P-labelled insert of cloned HBV-DNA as a probe. The sensitivity of this method is 0.1 pg of HBV-DNA, as reported previously [Pontisso et al., 1993].

Serum HCV-RNA

Serum HCV-RNA was evaluated by nested PCR using two sets of primers specific for the 5′ untranslated region (5′UTR) of the HCV genome, as described recently [Tisminetzky et al., 1994]. The sensitivity of this assay, in which 3 μl of serum are processed directly without RNA extraction, has been calculated at 10^4 genome Equivalents/ml (Eq/ml) [Pontisso et al., 1995a]. To identify patients with low levels of serum HCV-RNA, those negative by the above procedure were also studied after nucleic acid extraction from 100 μl of serum that were treated with 1 ml RNAzol solution (2 M/l guanidium thiocyanate, 12.5 M Na citrate [pH 7], 0.25% N-laurylsarcosine, 0.05 M/l 2-mercaptoethanol, 100 mM/l Na acetate [pH 4.0], and 50% water-saturated phenol.) After two extractions with chloroform, RNA was precipitated by chilled ethanol, washed with 70% cold ethanol solution, and resuspended in 20 μl of diethylpyrocarbonate-treated distilled water. This procedure was 10–100 times more sensitive than direct extraction starting from 3 μl of serum in detecting HCV-RNA, as deduced by serial dilution experiments carried out in parallel with serum and extracted nucleic acids of polymerase chain reaction (PCR)-positive control sera.

HCV Genotypes

To identify different HCV genotypes, an assay standardised in our laboratories and based on the hybridisation of the 5′UTR-amplified products with type-specific probes was used [Tisminetzky et al., 1994, 1995]. Briefly, PCR products were spotted in triplicate on nylon filters (Pall, Byodine B) and hybridised with fluoresceine-dUTP-labelled probes specific for HCV-1, HCV-2, and HCV-3, respectively. The results were then revealed by autoradiography, using an enhanced chemiluminescent method (ECL, Amersham International plc, England) [Pontisso et al., 1995a].

In patients reacting with the HCV-1 probe, subtypes 1a and 1b were defined by BstU I digestion of 5′UTR-amplified products. Since two restriction sites in HCV-1b and one restriction site in HCV-1a were present, after digestion and electrophoresis on acrylamide gel three fragments of 137 base pairs (bp), 30 bp, and 44 bp for HCV-1b and two fragments of 167 and 44 bp for HCV-1a could be generated, respectively (Fig. 1). The system has been validated previously by direct sequencing and by Line probe assay (LiPa, Immunogenetics, Belgium) [Pontisso et al., 1995b].

Statistical Analysis

Fisher's exact test and Student's t test were used for statistical evaluation.

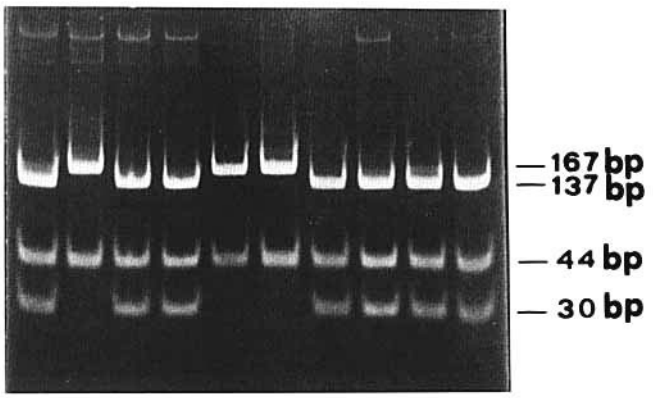

Fig. 1. Example of acrylamide gel electrophoresis after digestion with BstU I for subtype definition in patients found HCV-1 positive. 5′UTR-amplified products of patients with HCV-1a generate two fragments and those of patients with HCV-1b generate three fragments. On the right the fragment lengths are reported.

RESULTS

Virological Findings

Overall, 14 of 34 (41%) anti-HCV-positive and HBsAg-positive patients and 42 of 49 (86%) anti-HCV-positive patients without HBV infection had detectable serum HCV-RNA by nested PCR using 3 μl of serum ($P < 0.0001$). Seven additional cases (five anti-HCV/HBsAg-positive and two anti-HCV-positive/HBsAg-negative patients) became positive after nucleic acid extraction from 100 μl of serum. Among the 19 HCV-RNA-positive patients with dual HCV and HBV infection, 8 were infected by HCV-1 (3 by HCV-1a and 5 by HCV-1b), 10 by HCV-2, and only 1 by HCV-3 (Table I). All patients infected by HCV-1 had no detectable HBV-DNA in their serum and levels of HCV-RNA were $\geq 10^4$ Eq/ml, independently of the HCV-1 subtype. On the other hand, 5 of 10 patients infected by HCV-2 had levels of HCV-RNA lower than 10^4 Eq/ml ($P = 0.036$). Only 3 of the 19 HCV-RNA-positive patients were HBV-DNA positive, compared to 10 of the 15 remaining HCV-RNA-negative patients ($P = 0.003$), and all the 3 HBV-DNA-positive/HCV-RNA-positive patients were infected by the HCV-2 genotype. The patient infected by HCV-3 was positive for HCV-RNA while negative for HBV-DNA by spot hybridisation. Follow-up samples were available from several patients and most of them had a similar profile when tested serially. However, in a patient infected by HCV-1b and in whom a 14-year follow-up was available, serial serum testing demonstrated that the patient was HBV-DNA positive and HCV-RNA positive at clinical presentation and then cleared HBV-DNA from serum and HBeAg (anti-HBe seroconversion), while the patient continued to circulate HCV-1b (Fig. 2). This profile was associated with persistent evidence of liver disease.

Biochemical and Histological Severity of Liver Disease

Age, sex, and mean alanine aminotransferase (ALT) levels were not significantly different in patients in-

TABLE I. Clinical and Virological Data of 19 HBsAg-Positive/Anti-HCV and HCV-RNA-Positive Patients*

Genotype	HCV-1		HCV-2	HCV-3
	HCV-1a	HCV-1b		
No.	3	5	10	1
M/F	2/1	4/1	8/2	0/1
Mean age (years)	44	41	51	30
Range	27–47	30–56	22–64	
ALT (means ± SD)	156 ± 73	100 ± 52	124 ± 65	76
Histology				
CPH	1	2	2	1
CAH	1	2	3	—
Cirrhosis	1	1	5	—
HBeAg positive	—	—	3[a]	—
Anti-HBe positive	1	5[a]	6	1
e/anti-HBe negative	2	—	1	—
HBV-DNA positive	—	—	3	—
HCV-RNA $\geq 10^4$	3	5	5	1
		$P = 0.036$		

*CPH = chronic persistent hepatitis; CAH = chronic active hepatitis.
[a]One anti-HDV positive.

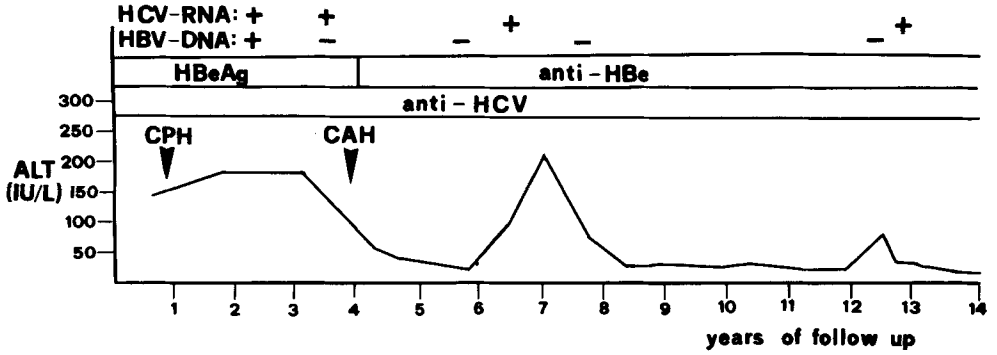

Fig. 2. Clinical and virological behaviour of a patient with HBV and HCV (genotype HCV-1b) coinfection during a follow-up period of 14 years.

fected by different genotypes although the mean age was higher in HCV-2 infected patients (Table I). Interestingly, in the patients with dual HCV and HBV infection, 5 of 7 patients with cirrhosis were infected by HCV-2, while 6 of 12 patients without cirrhosis had HCV-1, mainly 1b, at variance with the general findings in patients with HCV infection alone, where an association between HCV-1 and cirrhosis has been described [Dusheiko et al., 1994]. Of the seven patients with cirrhosis, two patients infected by HCV-2 had HCV-RNA levels $<10^4$, one being HBV-DNA positive.

DISCUSSION

Previous studies indicate that the presence of HCV in HBV-infected patients exerts a suppressive effect on HBV replication and may contribute to clearance of HBs antigenaemia [Liaw et al., 1991; Sheen et al., 1992]. On the other hand, it has been shown that in patients with both HBV and HCV infection, liver disease may be more severe in those with ongoing HBV replication, compared to patients without HBV-DNA but positive for HCV-RNA [Fong et al., 1991; Pontisso et al., 1993]. In the present study, an inverse relation between the replicative pattern of the two viruses has been observed. The mechanisms influencing replication of both viruses, with a reciprocal inhibitory effect, are still unknown. The results indicate that in HBsAg-positive patients with HCV infection supported by genotype 1, either 1a or 1b, HCV-RNA is always detectable in the serum and HBV replication seems to be inhibited by the concurrent virus. Another explanation might be that in these cases HCV could have superinfected healthy HBsAg carriers. However, the observations reported previously [Sheen et al., 1992; Liaw et al., 1994], that HCV superinfection in chronic HBV infection can induce not only HBV clearance but also termination of HBs antigenaemia, support the concept of a direct inhibitory role of HCV on HBV replication. The single patient described in our series, in whom HBV and HCV were replicating actively at presentation and hepatitis B clearance with anti-HBe seroconversion occurred early during follow-up, is in keeping with these findings. Disease activity seems to be supported therefore by HCV in these patients, at variance with patients infected by HCV-2, for whom active HBV replication can contribute to the severity of liver damage. Liver

cirrhosis has been indeed found frequently in this last group of patients, although only one showed active HBV replication. It should be pointed out that patients included in the present study were selected on the basis of HCV-RNA positivity in serum: It would be interesting to characterise liver HCV-RNA in those found HBV-DNA positive/HCV-RNA negative in serum, in order to verify whether they are infected by subliminal levels of HCV-2, as it has been described previously that HCV-RNA is detectable in the liver of such patients [Pontisso et al., 1993].

The findings may reflect a different efficiency of the transcription/translation machinery or sensitivity to endogenous cytokines induced by the concomitant virus, the final effect being different when HCV-1 or HCV-2 genotypes are responsible for the infection in the presence of HBV.

It has been reported that in a cultured cell system the N-terminal 122 amino acid residues of the core protein of HCV exert a suppressive effect on HBV gene expression and replication [Shih et al., 1993]. On the other hand, it is known that HCV-2 is more sensitive to interferon than HCV-1 [Yoshioka et al., 1991; Tsubota et al., 1994; Chemello et al., 1994; Pontisso et al., 1995a] and endogenous cytokines induced by replicating HBV [Pignatelli et al., 1986] may be responsible for the partial suppression of HCV-2 replication. In addition, HCV-1, at variance with HCV-2, induces strong activation of the hepatic interferon system [Ballardini et al., 1995] while it may be insensitive to its effect and this can efficiently concur with the clearance of HBV. Further studies, based on immune-histochemical and molecular techniques of liver samples, could be useful to clarify these events.

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